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Pursuing proteins in Parkinson's disease

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CONCLUSIONS AND GENERAL DISCUSSION



INTRODUCTION

Biomarkers are objective measures of a disease state and can be classified into different types, based on their application. Diagnostic biomarkers may help to improve the accuracy of a diagnosis of PD, preferably in the earliest stages when symptoms can be subtle and resemble clinically overlapping disorders. Progression biomarkers reflect the severity of the underlying disease process and can be used to carefully evaluate trial medication. Prognostic biomarkers may serve as markers of clinical subtypes associated with a known disease course. These biomarkers can be used for providing correct prognostic information to patients. In spite of major efforts that have been put into the search for PD biomarkers, the perfect biomarker has yet to be identified.

The main objective of this thesis was to identify candidate biomarkers for PD in lumbar CSF. A targeted approach was applied to evaluate the ability of selected CSF proteins in distinguishing PD patients from neurologically healthy controls, and to analyse their relationship with disease duration and severity. In parallel, we used an untargeted discovery approach to explore the proteome of affected PD brainstem tissue and CSF. The latter approach was aimed at identification of fully *novel* biomarker candidates for PD.

In our pursuit of PD biomarker candidates, our search strategy was based on the assumption that the most promising biomarker candidates are those that not only have changed expression levels in CSF of PD patients compared to healthy controls, but in addition reflect aspects of PD pathogenesis and/or have altered expression levels in brain tissue.

In this chapter, we will summarize and discuss our main findings. We will evaluate the strengths and weaknesses of our search strategy and elaborate on future steps necessary to bring promising candidate biomarkers towards clinical practice.

KNOWN BIOMARKER CANDIDATES

In **chapter 2**, we reviewed the literature to identify the most promising CSF biomarker candidates for PD. Although a large number of proteins and peptides had already been studied, mainly using a targeted approach, many proteins had been studied only once and still required subsequent validation in independent cohorts. We identified potential biomarker candidates based on the criteria that their expression in PD patients had to be different from controls in both CSF and brain tissue, and that a relationship with a pathogenetic mechanism was already known. The list of proteins that fulfilled these criteria included the oxidative stress related proteins ceruloplasmin, DJ-1 and oxidatively modified superoxide dismutase 1 (SOD1), the lysosomal enzyme beta-glucocerebrosidase, and proteins involved in protein aggregation and Lewy body formation: alpha-synuclein, tissue transglutaminase and osteopontin. A group of proteins involved in inflammatory processes and glial activation completed the list.

Since the publication of our review, other proteins have been identified that could be added to the list of potential biomarker candidates for PD, in particular alternative forms of alpha-synuclein, i.e. phosphorylated and oligomeric alpha-synuclein. These alpha-synuclein forms may either promote fibril formation (phosphorylated alpha-synuclein), or act as potentially toxic intermediates (alpha-synuclein oligomers) [1]. Increased levels of phosphorylated and oligomeric alpha-synuclein have been reported in both brain tissue [2,3] and CSF [4,5] of PD patients compared to controls. Further validation of these new biomarker candidates is necessary. In addition, some biomarkers already on the list have meanwhile been validated in independent cohorts. For example, several studies including our own (**chapter 3.1**), have confirmed that alpha-synuclein levels are reduced in PD patients [4-8].

In **chapter 3**, we analyzed the potential of a number of known candidate biomarkers that fulfilled the selection criteria described in chapter 2 to distinguish between PD patients and neurologically healthy controls.

Alpha-synuclein in CSF is the most obvious biomarker candidate for PD as it may reflect alpha-synuclein aggregation in the brain. In **chapter 3.1**, we demonstrated that, compared to neurologically healthy controls, CSF alpha-synuclein levels in PD patients are reduced. Although a reduction of CSF alpha-synuclein may seem counterintuitive, CSF levels could theoretically be reduced as a result of sequestration of pathological alpha-synuclein in the brain [9]. A large overlap in CSF alpha-synuclein levels was observed between PD patients and neurologically healthy controls. This implies that alpha-synuclein cannot suffice as a *single* diagnostic biomarker, but may have diagnostic value when used in a panel. Our finding is in accordance with the results of several large cohort studies [5-7,10], which also indicated that specificity and sensitivity of CSF α -synuclein as a diagnostic marker for PD is low. These other studies showed that alpha-synuclein levels are reduced in synucleinopathies other than PD, i.e. DLB and multiple system atrophy (MSA) [5-7].

While a single previous study reported an inverse correlation between CSF α -synuclein levels and disease stage [11], in our cohort of 53 PD patients, alpha-synuclein levels were not associated with clinical (motor section of the Unified Parkinson's Disease Rating Scale; UPDRS-III [12]) or imaging (striatal dopamine transporter binding) measures of disease severity. However, both measures predominantly reflect the loss of striatal dopaminergic projections and not necessarily the extent or load of alpha-synuclein pathology in the brain. Therefore, we can not exclude the possibility that alpha-synuclein levels in CSF do reflect the progression of alpha-synuclein pathology in the brain [13].

The endolysosomal degradation pathway has been implicated in the degradation of aberrant alpha-synuclein [14-16]. Dysfunction of this pathway may, therefore, play an important role in PD pathogenesis. This notion is corroborated by the findings described in **chapter 3.2**. Activities of seven enzymes implied in the endolysosomal degradation pathway

were studied in 58 PD patients and 52 age-matched neurologically healthy controls. Total protein-normalized cathepsin E and β -galactosidase activities were significantly higher in PD patients compared to controls, whereas normalized α -fucosidase activity was reduced. Similar to alpha-synuclein levels in CSF, the range of endolysosomal enzyme activities largely overlapped between PD patients and healthy controls. Sensitivity and specificity levels of only 63 percent were reached, implicating that these enzymes cannot be used as single biomarkers in individual patients.

In contrast to the results of a study by Balducci et al. [17], we did not find a significant difference in CSF beta-glucocerebrosidase activities between PD patients and controls. This discrepancy is most likely explained by differences in the choice of the control group. While neurologically healthy controls were included in our own study, Balducci et al. [17] used patients diagnosed with other neurological diseases as controls. In such controls, one cannot exclude the possibility that endolysosomal enzyme activities are abnormal as a result of a neurological disease other than PD.

Confirmation of our findings in independent study populations, including PD patients, healthy controls and patients with other neurodegenerative diseases is necessary to further evaluate the potential of endolysosomal enzymes to serve as biomarkers in PD.

In **chapter 3.3**, we evaluated the potential of the multifunctional chaperone protein clusterin as a biomarker for PD. Clusterin fulfils the criteria for potential biomarker candidates described in chapter 2: Příkrylová Vranová et al. [18] have reported increased CSF clusterin levels in PD patients compared to controls, and clusterin immunoreactivity has been observed in cortical Lewy bodies [19]. Furthermore, genetic analysis has previously linked clusterin to PD by finding an association between PD and a specific single-nucleotide polymorphism in CLU, the clusterin encoding gene [20]. This association was most pronounced in PD patients with dementia. The strong relationship between clusterin and Alzheimer's disease (AD) [21] indicated that potential changes in clusterin might not be specific for PD. Therefore, we measured CSF clusterin levels along with CSF levels of the AD related proteins amyloid- β -42, total tau and phospho-tau in a cohort of 52 PD patients and 50 age-matched neurologically healthy controls. Furthermore, we measured clusterin levels in plasma samples, which can be more easily obtained than CSF.

In accord with Lidström et al. [22], but in contrast to the results of Příkrylová Vranová et al. [18] who showed increased CSF clusterin levels in PD, neither CSF nor plasma clusterin levels differed between PD patients and controls in our study. Furthermore, clusterin levels in neither CSF nor plasma were associated with disease duration, disease stage (Hoehn and Yahr stage) or disease severity (UPDRS-III). These observations indicate that clusterin is not a suitable biomarker candidate for PD. It is noteworthy that we found positive correlations between CSF clusterin levels and the AD related proteins

amyloid- β -42, total tau and phospho-tau in both PD patients and controls. This may point towards an interaction between these proteins or a relationship with neuronal degenerative processes.

In summary, the findings in chapter 3 indicate that (activity) levels of some literature-based biomarker candidates differ between PD patients and controls. These include alpha-synuclein and endolysosomal enzymes, but not clusterin. Unfortunately, sensitivity and specificity for the discrimination between patients and controls was low for both alpha-synuclein and endolysosomal enzymes. It is therefore unlikely that these markers can serve as single diagnostic biomarkers. Nonetheless, they have diagnostic value when used in a panel of biomarkers.

NOVEL BIOMARKER CANDIDATES

The untargeted discovery approach of proteomics that we applied in the two studies described in **chapter 4** enabled the screening of large numbers of potential candidate protein biomarkers.

In the first study, reported in **chapter 4.1**, we compared the proteome of the locus ceruleus (LC) – an early-affected brain region in PD – of six PD patients and six neurologically healthy control brain donors. We studied whether differentially expressed proteins were related to previously identified or potentially unknown pathogenetic processes in PD. These differentially regulated proteins might ultimately serve as biomarker candidates. The most significantly deregulated proteins – tyrosine hydroxylase and dopamine beta-hydroxylase – reflect catecholaminergic neuronal loss in the LC of PD patients. Other previously implicated pathways and processes were reflected in the LC proteome of PD patients as well, most importantly mitochondrial dysfunction, oxidative stress and cytoskeletal dysfunction. One of the most interesting observations was the difference in expression levels of three aminoacyl-tRNA-synthetases (ARSs). ARSs are enzymes responsible for the first step of protein synthesis. They join amino acids to their cognate tRNAs [23,24] and are responsible for the incorporation of the correct amino acids in proteins. Since incorporation of the wrong amino acid can result in protein accumulation and cell death [25], there may be a pathogenetic link between ARSs and protein aggregation in PD. The potential role of aminoacyl-tRNA-biosynthesis in PD pathogenesis remains to be evaluated in future functional studies.

Among the almost 2500 proteins identified in the LC, 435 proteins were subsequently also detected in CSF samples of 10 drug-naïve PD patients and 10 neurologically healthy controls (discovery cohort; **chapter 4.2**), which accounts for 17% of the LC proteome (figure 1). For comparison, in a study by Pan et al. [26], a fraction of 9% of proteins identified in cerebral cortex tissue was present in the CSF proteome.

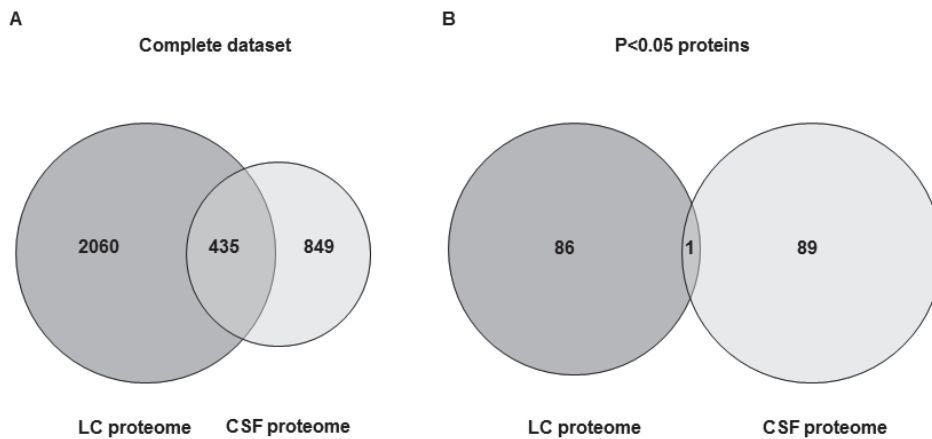


Figure 1: Venn diagrams of (A) all proteins and (B) deregulated proteins identified in the LC and CSF proteomics studies

The large fraction of blood-derived proteins in CSF – around 80% of the CSF proteome [27] – is one of the potential explanations for the limited overlap between the LC and CSF proteome. Another factor may be that not all brain-derived proteins identified in CSF will be present in a single specific brain region, such as the LC. As the proteome will most likely vary from one brain region to another, some brain-derived proteins in CSF will originate from other brain regions [26]. Finally, no proteome detected with the current state-of-the-art methods can be considered complete. Proteins with low concentrations might not have been detected.

Although pathogenetic processes related to the identified deregulated proteins overlapped between the CSF and LC study (complement activation, inflammation and axon guidance), only a single deregulated protein, i.e. regucalcin, identified in LC tissue was also deregulated in our CSF study (p-value below 0.05). Regucalcin levels were increased in LC tissue of PD patients compared to controls. In CSF, this protein was detected in none of the PD patients, but in 3 out of 10 controls, indicating low CSF concentrations already in controls, which questions regucalcins' biomarker value. Regucalcin plays a role in maintaining intracellular calcium homeostasis by activating mitochondrial Ca^{2+} pump enzymes [28]. Its neuronal expression is decreased in the cerebral cortex and hippocampus of the rat brain during aging [29]. According to our knowledge, regucalcin has not directly been linked to PD. Yet, a general association between calcium homeostasis and PD pathogenesis has been suggested based upon the possible neuroprotective role of centrally acting L-type calcium channel blockers in PD [30].

Other proteins that were deregulated in CSF of PD patients (but not in LC tissue) compared to controls included ribonuclease 4 (RNASE4; up-regulated in CSF of PD patients)

that catalyzes RNA degradation but also has neuroprotective properties and Ephrin type-A receptor 4 (EPHA4; down-regulated in CSF of PD patients), a receptor related to neurogenesis. Some of the deregulated proteins have previously been linked to PD, such as chromogranin A (CHGA).

When designing our studies, our assumption was that the most promising biomarker candidates would be those that have altered expression levels in both CSF and brain tissue of PD patients and, in addition, are associated with pathogenetic mechanisms. Therefore, we initially planned to select potential biomarker candidates for further analysis that would be deregulated in both our CSF and LC proteomics study. However, only a single protein (with very low spectral counts in CSF) was deregulated in both studies. Deregulation in our proteomics study in affected PD brain tissue did not prove useful as a selection criterion for CSF biomarkers.

A number of reasons may explain the minimal overlap between deregulated proteins in the LC and in CSF. First, deregulated proteins in CSF may be derived from other affected brain regions than the LC. Furthermore, not all proteins that are deregulated in the LC may end up in the CSF. Lastly, deregulated proteins may remain undetected due to incomplete detection of low-abundant proteins.

As a consequence of the minimal overlap between deregulated proteins in the LC and CSF, we decided to prioritize diagnostic biomarker candidates for PD using an alternative approach. We chose to focus on CSF and performed an overlap analysis with an independent CSF cohort of 12 PD patients and 13 controls (verification cohort). Application of the same proteomics workflow to this cohort confirmed the biomarker potential of three CSF candidates: myelin protein P0 (MPZ; up-regulated in PD), plastin-2 (LCP1; down-regulated in PD) and acid sphingomyelinase-like phosphodiesterase 3b (SMPDL3B; down-regulated in PD). In particular SMPDL3B, a protein involved in the degradation of sphingomyelin, is an interesting candidate. Previous studies have shown that expression of sphingomyelin is elevated in PD brains [31] and a mutation in another sphingomyelin phosphodiesterase, SMPD1, was recently identified as a strong risk factor for PD in two patient cohorts of Ashkenazi Jewish ancestry [32].

The overlap of deregulated proteins between the two CSF cohorts was limited. The most important potential explanation is the difference between the two patient groups, i.e. early-stage drug-naïve versus medicated PD patients with longer disease durations. As a result, potentially valuable markers may not have been confirmed in the verification cohort. Therefore, the corresponding biomarker candidates were not considered the only potential biomarkers that could be derived from our study. Non-overlapping proteins in the discovery dataset that well separate drug-naïve PD patients and controls, were directly or indirectly related to PD or a PD pathogenetic process and/or were part of an overlapping pathway or

subnetwork between the two datasets, such as EPHA4, CHGA and RNASE4, might also be promising candidate biomarkers. Though, confirmation must await a more extensive further validation in CSF of independent PD cohorts. For the overlapping CSF candidates, a relationship with PD pathogenesis needs to be proven with, for example, functional and/or neuropathological studies.

FURTHER STEPS TOWARDS CLINICAL APPLICABILITY

Neither the *novel* biomarker candidates that were identified in **chapter 4.2**, nor the studied literature-based and pathogenesis-related biomarker candidates described in **chapter 2 and 3**, are directly applicable in routine clinical practice. These candidates will need further evaluation and validation in larger numbers of CSF samples obtained in independent patient cohorts and – to validate the assays – in independent laboratories.

To evaluate the potential of CSF proteins as *diagnostic* biomarkers, validation cohorts should ideally include patients with atypical forms of parkinsonisms as well, as it is the discrimination of PD from other parkinsonian syndromes such as MSA, corticobasal degeneration and PSP, that has proven particularly difficult in early disease stages [33]. Moreover, the diagnostic potential of CSF biomarkers should be verified in prospective studies that include patients with parkinsonism at very early disease stages. Ideally, diagnoses should be confirmed neuropathologically. In addition, potential influential preanalytical factors should be evaluated in the further validation process, such as blood contamination, effect of circadian rhythms, pharmacotherapy and the possible influence of comorbid diseases.

Neurodegenerative disorders such as PD, MSA and PSP have various neuropathological characteristics and pathogenetic mechanisms in common. For example, much like PD, both MSA and PSP are neuropathologically characterized by the deposition of protein aggregates. Moreover, protein aggregates in both MSA and PD are principally composed of aberrant alpha-synuclein, even though the aggregates are found in oligodendroglial cells and neurons, respectively [34]. The neuropathological overlap between neurodegenerative disorders is reflected in CSF protein levels. For example, total CSF alpha-synuclein levels are decreased in both PD and MSA patients [5-7]. Similarly, markers that reflect inflammatory processes are increased in both PD and PSP patients relative to controls [35]. Lastly, intracellular-derived CSF proteins, such as neurofilament proteins, are increased in both MSA and PSP [6], diseases that tend to progress more rapidly than PD. However, these proteins do not discriminate PD patients from controls. In the end, it may therefore prove difficult to identify any single CSF biomarker that is exclusively disease-specific. Further validation studies will clarify the disease-specificity of the novel candidate biomarkers of our proteomics study in CSF.

The use of a panel of CSF biomarkers may provide a solution. Combining biomarkers into a panel could address the neuropathological and pathogenetic overlap and reach sufficiently high sensitivity and specificity for an accurate discrimination of PD patients from both healthy controls and other neurodegenerative disorders. In AD, a panel of four CSF biomarkers, i.e. amyloid- β -42, alpha-synuclein, total tau and phospho-tau can be used to differentiate between patients with AD and patients suffering from DLB or PD related dementia (PDD) [6]. In a similar way, a combination of two or more CSF biomarkers might also work for PD. This type of combination may, for instance, include CSF levels of various forms of alpha-synuclein (i.e. total, phosphorylated and/or oligomeric alpha-synuclein), endolysosomal enzyme activities and one of the novel biomarker candidates identified in our CSF proteomic study, combined with a measurement of neurofilament proteins to differentiate PD patients from the atypical parkinsonian disorders MSA and PSP [6].

In our studies, we aimed to identify proteins that could differentiate PD patients from controls, which could be seen as a first step in the identification of *diagnostic* biomarker candidates. Consecutive validation in larger patient cohorts may indicate that specific biomarker candidates derived from our study may also reflect the clinical phenotype (i.e. *progression* and *prognostic* biomarkers). Prospective longitudinal studies are required to identify *prognostic* biomarkers. Studies designed to evaluate a relation with disease severity should preferably be performed in cohorts that include patients with a wide range of disease severity. Subsequently, the markers' ability to reflect disease progression could be evaluated with longitudinal measurements. A good example of this type of study design is the study by Shi and colleagues [36]. The CSF fractalkine-amyloid- β -42 ratio was identified as a potential marker of disease severity and disease progression. This ratio positively correlated with UPDRS motor scores and Hoehn and Yahr stages in cross-sectional samples as well as with the annual rate of UPDRS motor score progression in longitudinal samples.

CONCLUDING REMARKS

In this thesis, we have shown that levels of alpha-synuclein and endolysosomal enzyme activities in CSF differ between PD patients and controls. The untargeted proteomics studies in CSF and brain tissue provided novel biomarker candidates as well as insight into PD pathogenesis. Independent large-scale validation studies are required to confirm our findings and to evaluate the potential of the candidate biomarkers to discriminate PD from other neurodegenerative disorders, to distinguish between PD subtypes, or to serve as disease progression markers. The odyssey of pursuing the most effective CSF biomarkers for PD will certainly be continued after the publication of this thesis.

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